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Long oligonucleotide microarrays in wheat: evaluation of hybridization signal amplification and an oligonucleotide-design computer script

D. Skinner (✉)
USDA-ARS and Crop and Soil Science Department
209 Johnson Hall
Washington State University
Pullman, WA 99164, USA
e-mail: dzs@wsu.edu

P. Okubara
USDA-ARS and Plant Pathology Department
367A Johnson Hall
Washington State University
Pullman, WA 99164, USA

K-H Baek
Crop and Soil Science Department
211 Johnson Hall
Washington State University
Pullman, WA 99164, USA

D. Call
Department of Veterinary Microbiology and Pathology
415 Bustad Hall
Washington State University
Pullman, WA 99164 USA

Abstract

A computer script was written in the Perl language to design equal-length long oligonucleotides from DNA sequences. The script allows the user to specify G+C content, melting temperature, self-complementarity, the maximum number of contiguous duplicate bases, whether to start with the first start codon and whether to report reverse-complements. Microarrays were fabricated with 95 oligonucleotides (60 mers) representing 41 genes. The microarray was interrogated with cDNA from roots and shoots of two near-isogenic lines and a commercial cultivar of *Triticum aestivum* L. (hexaploid wheat) challenged with cold temperature, hot temperature, or the biological control bacterium *Pseudomonas fluorescens.* Self-complementarity of the oligonucleotides was negatively correlated with signal intensity in 23 of 54 arrays (39%; $P < 0.01$). Tyramide signal amplification was essential for signal generation and detection. Genes involved in signal transduction pathways responded similarly following exposure to cold, heat and *P. fluorescens*, suggesting intersection of the pathways involved in response to these disparate stress factors. Microarray results were corroborated by quantitative real-time PCR in 75% of samples assayed. We conclude that long oligonucleotide microarrays for interrogation with cDNA from hexaploid wheat should be constructed from oligonucleotides having minimal self complementarity that also meet user-specified requirements of length, G+C content and melting temperature; multiple oligonucleotides should be used to represent each gene; and Tyramide signal amplification is useful in wheat oligonucleotide microarray studies.

Keywords: *Triticum aestivum* • Tyramide • Microarrays • Oligonucleotide

Introduction

The long term goal of our work is to identify wheat genes that mediate root responses to multiple abiotic and biotic stresses, with the view to applying fundamental knowledge of stress signaling to molecular breeding. Hexaploid wheat (*Triticum aestivum* L.) is one of the world's most important staple crops, and supports a $7 billion industry in the US. Wheat varieties are sown either in late fall or in spring, depending upon a genetically determined "winter" or "spring" growth habit. The winter varieties require a period of prolonged cold (vernalization) for flowering, a trait that has been mapped to a single locus in each genome of hexaploid wheat (Sutka et al., 1999; Yan et al., 2003). Regardless of growth habit, both winter and spring wheat face similar stresses during the growing season. For instance, acute cold temperature can cause stress to both winter and spring wheat, and can especially impact spring varieties following planting. In some wheat-growing regions, high temperature is a source of damaging stress, particularly during anthesis or grain filling stages of growth.

Persistent sources of biotic stress to roots include fungal root pathogens, such as *Rhizoctonia solani*, *R. oryzae*, *Pythium* spp., and *Fusarium* spp., and possibly, the biological control bacteria that suppress these pathogens. One such biocontrol bacterium is *Pseudomonas fluorescens*, which exerts antifungal effects by a variety of

mechanisms, including production of antifungal metabolites (Weller et al., 2002; Haas and Keel, 2003), and induction of defense responses in the foliage (systemic resistance) (Hase et al., 2003). Strains of the D genotype (Mavrodi et al., 2001) are especially effective in controlling the fungal root pathogen *Gaeumannomyces graminis* var. *tritici*.

Microarrays are currently the most widely used tool for simultaneously comparing changes in the expression of large collections of genes, including genome-scale data collections, and have revealed complex patterns of coordinated gene expression in plants (e.g., Glazebrook et al. 2003; Kreps et al. 2002; Seki et al. 2002). The probes used in microarrays may be cDNAs derived from transcripts, or they may be 50-70mer (long) synthetic oligonucleotides. Long oligonucleotides are advantageous in that they can be designed to represent divergent regions of highly similar genes, such as members of a gene family, and they can be designed from publicly available sequences, obviating the need to clone or amplify DNA segments.

Bozdech *et al*. (2003), Rouillard *et* al. (2002), and Li and Stormo (2001), describe programs used to design oligonucleotides to represent entire genomes. Those programs were designed to discover oligonucleotides that are uniquely represented in the genome and thus require sequence information for all genes of the organism under study. Nielson *et al.* (2003) described "Oligowiz" which runs on a remote server (or on a local server under a licensing agreement) and designs oligonucleotides for a set of sequences the user must upload to the server. Oligowiz, under the default settings, recommends a single oligonucleotide per sequence, and the oligonucleotides recommended are of variable length, having met other criteria that are examined in preference to oligonucleotide length. Herold and Rasooly (2003) describe a program that choses oligonucleotides from one or more DNA sequences; the program is oriented towards finding oligonucleotides to uniquely represent closely-related DNA sequences. Each of these programs is designed to provide a single oligonucleotide to represent each subject DNA sequence.

Our objectives were to develop a user-friendly computer script to design equal-length oligonucleotides, construct a small array of oligonucleotides representing genes from hexaploid wheat (*Triticum aestivum* L.), and evaluate the applicability of a signal amplification method to microarray interrogation with cDNA from hexaploid wheat. To test the performance of oligonucleotides that were designed using Olinuc, we interrogated our test array with cDNA populations from seedling roots and shoots after cold or hot temperature treatments, and after root colonization by the biological control agent *Pseudomonas fluorescens* strain Q8r1-96 (Raaijmakers et al., 1999).

The 41 wheat expressed sequence tags (ESTs) and cDNA sequences selected for use in our array represent diverse cellular, metabolic, and physiological processes. For example, two encode transcripts regulated by the plant growth regulator abscisic acid, four encode seed storage proteins, six encode homologues of heat shock proteins, three are homologues of drought- or cold-induced genes in Arabidopsis, six are homologues of defense-related genes, and seven have putative roles in oxidative

stress. To identify possible constitutively-expressed controls, 15 oligonucleotide probes, representing eight genes that have roles in carbon metabolism and housekeeping, were included in the array. As negative hybridization controls, a total of 13 oligonucleotides (from six genes) were derived from antisense strands.

Experimental methods

A computer script, which we have named "Olinuc," was written in Perl Version 5.8, available from http://www.perl.org. The user interface was written using the Perl/Tk module graphical user interface tools. The level of self-complementarity was calculated as the maximum proportion of the oligonucleotide aligned with a complementary base when the oligonucleotide was aligned with itself. All possible alignments were evaluated. The percentage G+C was calculated as the total count of G and C residues divided by the total number of bases in the oligonucleotide. The melting temperature of the oligonucleotide was determined with the nearest neighbor method using the the equation:

$$T_m = \left\{ \frac{(\Delta H)1000}{A + \Delta S + R\ln(C_t/4)} \right\} (16.6\log_{10}[Na^+] - 273.15)$$

where ΔH and ΔS are the respective sums of the nearest neighbor enthalpy and entropy thermostability parameters provided by SantaLucia (1998), R is the universal gas constant (1.987 cal deg^{-1} mol^{-1}), C_t is the molar concentration of the oligonucleotide strands and A is a helix-initiation factor equal to –10.8. The concentrations of oligonucleotide strands and Na^+ were set to 0.5 µM and 100 mM, respectively.

Olinuc differs from earlier programs in that it allows the user to specify a subset of sequences from a master DNA sequence file and automatically checks for duplicate entries in the subset. Additionally, Olinuc gives the user the option of designing oligonucleotides from the entire sequence, or by restricting the analysis to sequence following the start codon, or from between the first start and first stop codons (coding region). Olinuc allows the user to specify the maximum allowed self-complementarity of the entire oligonucleotide, as well the maximum allowed size (as a percentage of the total) of shorter regions of 80% or more self-complementarity, and the maximum number of consecutive duplicate bases. From the results, the user may select as many overlapping or non overlapping oligonucleotides to represent each sequence as desired. Olinuc also identifies sequences for which no suitable oligonucleotides were found, and optionally, reports reverse-complements of oligonucleotide sequences that are useful as negative controls.

The microarray hybridization experiments are described to address the six sections of MIAME standards (Brazma et al. 2001).

1. Experimental design: overview of the hybridization experiments.

The experiment was designed to assess changes in gene expression in wheat seedling roots and shoots following a one-hour exposure to cold or hot temperature, or after four days of root colonization by *Pseudomonas fluorescens*, a bacterium known to suppress certain fungal root pathogens.

2. Array design

Arrays were constructed on epoxy-silanated glass slides following the methods of González et al. (2004). The slides were masked with Teflon to form 10 wells on each slide (part number ER-298, Erie Scientific Co., Portsmouth, NH, USA). Ninety-five oligonucleotides were designed for 41 genes (Table 1) using Olinuc. All of the oligonucleotides were selected from within the coding regions of the genes. The melting temperatures ranged from 71.9°C to 78.3°C. GC content ranged from 0.48 to 0.60, and the level of self-complementarity ranged from 0.2 to 0.53. Oligonucleotides were all 60 bases in length. At least two oligonucleotides were designed for each gene, and four to five were generated for six of the genes. Oligonucleotides representing the antisense strand of six genes were included as controls (Table 1).

Oligonucleotides were synthesized by Illumina, San Diego, CA, and diluted in print buffer (0.1 M Na2HPO4, 0.2 M NaCl, 0.01% sodium dodecyl sulfate) to a final concentration of 60 µM and spotted onto the slides in duplicate by using a MicroGrid II spotter (BioRobotics, Inc., Woburn, Mass.). Printed slides were baked for 60 min at 130°C in a vacuum oven and stored at room temperature. Each microarray included two spots of an arbitrary 25-mer biotinylated oligonucleotide that served as controls for detection chemistry and normalization. Two negative control spots consisting of only print buffer were also represented on each array for background subtraction. Each microarray was composed of a total of 194 spots deposited into each well of the slide.

3. Samples used:

Total RNA was extracted from three wheat lines, 442, 443 and cultivar 'Finley' as described below. Lines 442 and 443 are near isogenic lines derived from 'Centurk 78' X 'Norstar' and differed primarily at the vrn1A-Fr1 region of chromosome 5A (Storlie et al., 1998). Line 442 is a winter wheat, and 443 is spring wheat. 'Finley' is a hard red winter variety derived from different parental lines than 442 and 443 (http://www.ars-grin.gov/npgs/).

cDNA was constructed from RNA isolated after the following treatments (two to five replicates of each):

a. Cold - wheat seeds were germinated in darkness at 25^{o}C in plastic boxes on moist filter paper, allowed to grow for four days, exposed to 2^{o}C for 1 h, and harvested immediately after cold treatment.

b. Heat - wheat seeds were germinated and seedlings grown as above, then were harvested immediately after a treatment of 50^{o}C for 1 h.

c. Root-colonizing biocontrol bacteria - wheat seeds were coated with 10^5 CFU/ seed (Landa et al., 2002) of *Pseudomonas fluorescens* strain Q8r1-96 (Raaijmakers et al., 1999), germinated as above, and grown for four days prior to harvest.

Roots and shoots were separately collected, and immediately frozen in liquid nitrogen. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as follows. For cold and heat treatments, 100 mg of tissue was ground in liquid nitrogen using a mortar and pestle, and then covered with 1 ml TRIzol, which froze to the mortar surface. Upon thawing, the tissue in TRIzol was transferred to a 1.5 ml tube and subsequent extraction procedure followed manufacturer recommendations. The resulting mRNA pellet was dissolved in 50 μL DEPC-treated water; 4 μL were diluted into 396 μL of TE for spectrophotometric quantification, and 8 μL were analyzed by gel electrophoresis in 1X TAE, 0.8% SeaKem LE agarose (FMC) to assess the quality of the RNA. For seedlings undergoing root colonization by *P. fluorescens* Q8r1-96, root and shoot tissue was collected from 80 to 85 seedlings of cv. Finley, and 45 to 50 seedlings of 442 and 443 (approximately 2 g fresh weight). After pulverization in liquid nitrogen, tissue was transferred to a tube containing 10 to 20 mL of TRIzol, and extractions were carried out as recommended by Invitrogen. RNA quality was assessed by gel electrophoresis using 1% agarose containing 6% formaldehyde in MOPES buffer (Sambrook et al, 1989).

Total RNA (10 μg) was used as template in reverse transcription reactions. The composition of the synthesis buffer was: 2.5 μM oligo dT_{20} primers; 0.5 mM dATP, dTTP and dGTP; 0.375 mM dCTP; 0.06 mM biotinylated dCTP; 1X first strand buffer provided with the Superscript II enzyme; 10 mM DTT; 2U RNase Out, and 10U Superscript II. All reagents were obtained from Invitrogen Corp. Synthesis was performed in a thermocycler for 2 h at 42°C. The resulting cDNA-RNA complexes were precipitated in 0.3 M sodium acetate, pH 5.2 and 67% ethanol. Pellets were redissolved in 32 μL 1.6 mM EDTA, 39 mM NaOH, then heated at 72°C for 10 min to dissociate the DNA-RNA complexes. The mixture was centrifuged briefly then neutralized by adding 2.5 μl of 500 mM HCl and 2.5 μl of 500 mM TRIS, pH 7.0. The interrogation solution was generated by diluting this neutralized mixture 1:1 in 4X SSC, 5X Denhardt's solution.

4. Hybridization and signal amplification

The interrogation solution was heated at 95°C for two minutes and then applied to microarrays that had been prehybridized for 30 min with 100 mM Tris-HCl, 150 mM NaCl, 0.5% blocking reagent from Tyramide Signal Amplification (TSA™) kit #NEL-700A (Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA). Hybridization was allowed to proceed 16 to 18 h at 48°C. Slides were processed at room temperature for post-hybridization signal amplification using the biotin–based TSA kit. Three 1-min washes in TNT wash buffer (0.05% Tween 20, 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) were carried out immediately following hybridization and between each of the following incubations: 1) streptavidin-horseradish peroxidase conjugate from the

TSA™ kit diluted 1:100 in TNB buffer (0.1 M Tris-HCl, pH 7.5, 0. 15 M NaCl, 0.5% blocking reagent supplied in kit) for 30 min; 2) 10% bovine serum albumin, 2X SSC for 30 min; 3) Biotinyl Tyramide Amplification reagent, diluted 1:50 in 1X Amplification Diluent, for 10 min; 4) 1:200 dilution of 1 mg/ml streptavidin-Alexa Fluor 546® conjugate (#S-11225, Molecular Probes, Eugene, Oregon) diluted in 1X SSC, 5X Denhardts solution, 1 h. Following the last TNT wash, slides were dried by centrifugation at 200 *xg* for 2 min.

5. Measurements

Slides were scanned with a Genescan LSIV microarray reader (Genomic Solutions, Ann Arbor, MI). The Alexa Fluor 546® fluorophore emits light at the same wavelength as Cytidine-3, hence signal was detected using the Cy-3 channel. Images were partitioned using the "Integrator" scanner software supplied with the scanner and pixel totals were used in subsequent analyses. The gain was adjusted empirically such that the intensities of the biotin control spots were just under the maximum.

6. Normalization controls

Two blank spots included in each array were used to determine a background pixel value for each slide. The mean of the blank spots was subtracted from each feature pixel value on the array. The mean of the two biotin control spots in each array were normalized to the grand mean of all biotin spots in the entire experiment. The multiplication factor for this normalization for each array was then applied to all feature spots for that array. This procedure resulted in the normalization of the arrays such that the biotin controls all had the same mean pixel value and the blank spots were all set to zero, correcting for variation in hybridization and detection efficiency between arrays, but not for differences in labeling or cDNA synthesis efficiency. Correction for the latter two sources of variation requires standardization to a constant-expression oligonucleotide.

A constant-expression gene encompassing the treatments used here has not been determined prior to this study. However, two oligonucleotides representing wheat iron-superoxide dismutase (Fe-SOD; TIGR accession TC29145) consistently showed strong signal in all treatments, suggesting that this gene may be suitable as a spiking control. The biotin-normalized data signal level from these oligonucleotides was not significantly ($P > 0.05$) different in any of the arrays (analysis not shown). In an unrelated study using quantitative real-time PCR, we found that expression levels of Fe-SOD remained nearly constant throughout a 4-week cold acclimation period in 442 and 443 wheat (Baek and Skinner, 2003). Therefore, we used the mean signal of these two Fe-SOD oligonucleotides as a constant-expression control and normalized all other signal measurements to this mean.

The normalized pixel values were log (base 2)-transformed and hybridization to each oligonucleotide was represented by the mean pixel value of the two spots on the slide. Any normalized values less than zero were set to one prior to log2 transformation to

yield a value of zero after transformation. These values were then "centered", that is, the data values for each array were standardized to a mean of zero and a standard deviation of one. These data then were analyzed using "SAM" software (Tusher et al., 2001).

Quantitative RT-PCR (qPCR)

RNA was extracted using Trizol (Invitrogen, San Diego, CA) according to the manufacturer's instructions and stored at – 70 oC before being used for qPCR. The primers for manganese superoxide dismutase and glutathione peroxidase were constructed by using Primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi); Coi1 primers were designed using OLIGO 6.0 (Molecular Biology Insight, Inc., Cascade CO; on-line at Life Sciences Research, Long Lake, MN) Each primer was composed of about 20 nucleotides with melting temperatures around 60 oC. The primer sets were designed to produce an amplicon ranging from 90 to 110 nucleotides. Primer sequences are available from the first author on request. Complementary DNA was constructed as described above (without biotinylated dCTP) and 10ng were used as template in qPCR carried out in LightCyler (Roche Applied Science, Penberg, Germany).

Results

Oligonucleotide design script

Olinuc is accessed through a graphical user interface. This interface allows the user to specify oligonucleotide length, minimum and maximum G + C content, and minimum and maximum melting temperature. Selection of the coding region, limits to self complementarity, and number of consecutive duplicate bases are available through selection of advanced options. In an example of its use, application to 14 complete coding sequences from wheat resulted in the generation of 3,303 oligonucleotides meeting the criteria of 60 nucleotide length, 60-70% G+C, and Tm of 80-85°C. After the script deleted all oligonucleotides not meeting criteria of 40% maximum self-complementarity, regions of 80% or more self-complementarity limited to 10% of the oligonucleotide length, and a limit of five consecutive duplicate bases, 769 oligonucleotides remained; suitable oligonucleotides were found for all sequences. An example of the output of the script is shown in Fig. 1. The output provides the position within the sequence occupied by the oligonucleotide, allowing the user to easily select one or more overlapping or non-overlapping oligonucleotide(s). The output of Olinuc is written in FASTA format (Fig. 1), so that the user can directly submit the oligonucleotides for BLAST (Altschul et al., 1997) searches and to other computerized DNA analyses, if desired.

Reproducibility of signal intensities among biological replications

Arrays were interrogated with each cDNA (RNA) population two to five times. The correlation coefficients comparing replications within treatments ranged from 0.58 to 0.93 with a mean of 0.78. Each of these values was significant at $P<0.0001$, indicating

that our interrogation, normalization, and standardization methods resulted in consistent signal intensity across replications.

Effect of oligonucleotide self-complementarity on signal intensity.

The self complementarity of the oligonucleotides used in the array ranged from 0.20 to 0.53. Intensity of the signal from each oligonucleotide was significantly and negatively correlated with self-complementarity in 23 of 54 arrays analyzed (r between -0.24 and -0.35; $P<0.01$), indicating a weak and inconsistent relationship. Because this relationship was not statistically significant in 31 of 54 arrays, the effect of self-complementarity appears to be relatively minor in most cases.

Effect of signal amplification

To test the need for the Tyramide signal amplification step, six separate interrogating cDNA preparations were divided into two aliquots each and hybridized to two separate arrays. Following hybridization, the arrays were treated identically, except that the 10 min signal amplification step was omitted in one of the arrays. The results were unequivocal; arrays subjected to amplification yielded strong differential signal (Fig. 2), whereas the arrays without amplification yielded virtually no signal. In the latter, only the biotin controls were visible (not shown).

Significance analysis, identification of constitutively-expressed controls, and differentially-expressed genes

Expression level changes indicated as significant by one oligonucleotide from a given gene were not always corroborated by a second oligonucleotide from the same gene. However, there were no instances of contradictory results found in our limited array. Combined results from the oligonucleotides representing each gene are shown in Table 2. Differentially expressed genes were up-regulated to 1.1 to 3.1-fold or down-regulated to 0.3 to 0.8-fold (Table 2; determined with SAM software; Tusher *et al.,* 2001). Eleven genes responded similarly in the roots of NILs 442 and 443 to the heat exposure (Table 2). However, there were considerable differences in the responses of genes in shoot tissue to the heat shock. NIL 443 (spring wheat) responded with 13 up-regulated genes, while NIL 442 (winter wheat) responded with only two (Table 2).

A total of 20 genes were significantly up-regulated in shoot tissue in response to cold; 16 in the spring wheat but only 8 in the winter wheat; five genes were up-regulated in common in both NILs (Table 2). Surprisingly, no genes were significantly down-regulated in shoot tissue in response to cold temperature in either NIL (Table 2). Responses of genes in root tissue exposed to cold were essentially opposite to the response in shoot tissue. No genes were significantly up-regulated in the spring wheat NIL in response to cold, while 11 genes were down-regulated (Table 2). In the winter wheat NIL, 9 genes were down-regulated and nine genes were up-regulated (Table 2).

A total of 17 genes were indicated as significantly up- or down-regulated in response to

root colonization with *P. fluorescens* in 442, 443, or Finley wheat (Table 2). In general, the expression of these genes was similar in the three wheat lines, with the exception of heat shock protein 26.6 and puroindoline A, which were up-regulated in one genotype but down-regulated in another (Table 2).

The most responsive gene was pMA1951, an abscisic acid-responsive transcript (GenBank accession U43718) which was up-regulated in six of the 11 genotype/treatment combinations (Table 2). Wheat EST BE445579.1, with extensive homology to the *Arabidopsis* jasmonate pathway signal component COI1 (AY168645.1), also was up-regulated in five of the 11 genotype/treatment combinations, as was alpha-amylase, GenBank accession M16991 (Table 2). Several other genes also showed significant up-regulation in response to all of the stress factors in at least one of the wheat genotypes (Table 2).

Relatively high proportions of the genes that responded to one stress factor also responded to another stress factor in at least one target RNA population (Table 3), probably indicating shared portions of the pathways involved in the early responses to the stress factors we investigated.

Two exceptional expression patterns were noted among our putative "negative" hybridization probes. Two oligonucleotides representing the human receptor–associated protein BAP 37 (GenBank accession AF126021) hybridized to transcripts from cold or heat-challenged plant tissues (Table 2). BLASTn searches revealed that these oligonucleotides have significant sequence identity (50% and 53.5%) to the maize prohibitin gene, suggesting that the observed signal was due to hybridization with wheat prohibitin cDNA. The wheat prohibitin gene has not yet been sequenced, but assuming a high level of similarity to the maize prohibitin gene, this result further suggested that the signal amplification method and the hybridization conditions we used allowed the detection of signal resulting from hybridization of 60-base oligonucleotides to targets with perhaps as little as 50% homology. An oligonucleotide representing human myosin light chain 2 (GenBank accession M21812) also resulted in measurable signal, and indicated significant down-regulation in root tissue exposed to heat or cold (Table 2). A BLASTn search indicated that this oligonucleoide shares about 50% identity with portions of several rice (*Oryza sativa*) BAC clones, and suggests that a gene with coincidental homology to myosin light chain is expressed in both wheat and rice.

Quantitative real-time PCR was used to seek corroboration of the microarray results. A total of 16 probe/target combinations were tested (Table 4). The SAM data analysis procedure is not applicable to qPCR data, hence, no direct comparison of significance levels between microarray and qPCR results was attempted. However, the two methods were consistent in 12 of the 16 probe/target combinations (differences are indicated in bold in Table 4).

Discussion

It has long been known that cold and heat stress elicit similar responses from plants. Plants that have been exposed to a heat shock exhibit enhanced cold tolerance (Jennings and Saltveit, 1994; Lafuente et al., 1991; Leshem and Kuiper, 1996; Lurie et al., 1994), and vice-versa (Fu et al., 1998). These results suggest that the mechanisms underlying the response to these diverse temperatures are very similar. Large scale evaluations of gene expression in Arabidopsis have shown that while the initial (3 hours) transcriptional regulation changes in response to heat and cold were similar, they quickly diverged until very few genes were behaving similarly after 27 hours (Kreps et al., 2002). It also was observed that the gene expression behavior was very different in roots compared to leaves (Kreps et al., 2002), indicating another level of specificity of response.

Microarray studies on cereal gene expression level changes in response to stress have been carried out in barley (Atienza et al., 2004) and rice (Rabbani et al., 2003). Both studies used cDNA microarrays. The study on barley examined transcript level changes in response to cold, heat, dehydration, high light or copper stress in the shoots of seedlings (Atienza et al., 2004), while the rice study apparently considered whole seedlings (roots and shoots combined) after 5, 10 or 24 hr. of cold, drought or high salinity stress and/or ABA treatment. The results we have reported here appear to be the first to use long oligonucleotide microarrays to investigate the response of cereal root and shoot tissue separately, and to include rhizobacteria colonization as a stress factor.

The gene regulation information we have obtained from long oligonucleotide microarrays of wheat indicated that the spring and winter wheat NILs responded very differently to temperature stress. The response to heat stress can be summarized as follows: a greater number of genes responded in shoot tissue of the spring wheat NIL than in that of the winter NIL, while the roots responded similarly in both NILs. The response to cold stress can be summarized as follows: the root tissue is much more transcriptionally active in the cold response of the winter wheat NIL than the spring wheat NIL, while the shoot tissue is much more active in the spring wheat NIL than in the winter wheat NIL.

In root tissue, three genes showed similar expression changes in response to cold stress, heat stress, and *P. fluorescens*, suggesting that the pathways involved in response to these disparate stresses intersect at key points or share common components. Because at least one of these genes is known to be involved in signaling, we suggest that expansion of the long oligonucleotide microarray analysis we have conducted here will lead to an understanding of the pathways involved in the response of wheat to both biotic and abiotic stress factors.

Although previous studies of gene transcript level changes in cereal roots responding to rhizobacteria colonization have not been done, extensive microarray-based studies have been conducted to examine changes in expression of host genes during colonization of Arabidopsis roots by Pseudomonas spp. In an Arabidopsis-P. thivervalenesis interaction, only a handful of root-expressed transcripts underwent

changes in steady state levels. Of 14,300 genes in the array, nine were differentially regulated, and all were repressed (Cartieux et al., 2003). In contrast, we observed two genes, COI1 and puroindoline B, that were positively-regulated during root colonization. The absence of significant down-regulation in our interaction could be attributed to the very modest size of our array. Transcriptome profiling of the Arabidopsis-P. fluorescens interaction revealed that 97 transcripts were differentially regulated in roots at 3 and 7 dpi (Verhagen et al., 2004). Thirty nine of these, including five involved in defense and stress, were induced. None corresponded to COI1 or puroindoline B. Transcripts encoding positive regulators of the ethylene signal pathway, peroxidases, and kinases were repressed in roots (Verhagen et al., 2004). The authors noted that the host ecotypes used in each study differed at the ISR1 locus (Ton et al., 2002) and, hence, in responsiveness to jasmonate and ethylene, and in innate immunity to necrotrophic pathogens. There is no clear indication yet which of the two Arabidopsis-rhizobacteria interactions, if either, our system will more closely resemble.

Reduced signal intensity as a function of self-complementarity of the oligonucleotides was observed and may have resulted from formation of secondary structure of the oligonucelotides, from interaction of multiple oligonucleotides during deposition onto the slides, or from secondary structure formation of the interrogating cDNA during the hybridization process. To avoid attenuation of signals, use of oligonucleotides having the lowest possible degree of self-complementarity and otherwise meeting criteria of length and melting temperature is recommended.

The Tyramide amplification step was essential for the detection of signals from long oligonucleotide probes in our hexaploid wheat arrays. Whether this step can be eliminated for highly abundant transcripts or for populations of lower sequence complexity remains to be tested. Some but not all oligonucleotide probes that represented the same signature stress homologue gave the expected trend in gene expression. These divergent results might be attributed to differences in physical characteristics, such as melting temperature or degree of self-complementarity. Signal intensity for an oligonucleotide that is derived from a conserved domain is expected to be higher than that from a unique sequence if multiple genes containing that domain are expressed in the same cDNA population. Because not all oligonucleotides designed from the same sequence performed identically, we recommend that at least two oligonucleotides be used for each sequence of interest.

The microarray results we obtained were corroborated by qPCR in 75% of the probe/target combinations examined. It recently was demonstrated that microarray results can differ dramatically between platforms, with as little as 2% concordance among three systems (Marshall, 2004). Also, because we studied allohexaploid wheat (three ancestral genomes), it is possible that the qPCR primers we used primarily amplified a different gene from that detected by the oligonucleotide probes used for the microarray analysis. Hence, it is possible that 75% concordance between the two expression level measurement systems (qPCR and long oligonucleotide microarray) will be commonly found.

Olinuc provides a simple and convenient means to generate multiple long oligonucleotides from sequences of interest to the user. The flexibility provided in the graphical user interface allows the user to specify numerous characteristics for the oligonucleotides and to easily modify these criteria. Olinuc and the hybridization and signal amplification conditions described here provide useful tools for investigating gene expression regulation in hexaploid wheat. Olinuc is available from the first author on request.

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Figure Legends

Figure 1. Example of output from a Perl script used to design oligonucleotides from DNA sequence data. The position of the oligonucleotide within the sequence, the melting temperature, the G+C proportion of the oligonucleotide and the level of self-complementarity is provided for each oligonucleotide.

Figure. 2. Typical hybridization obtained with 60 mer oligonucleotide probes. 1, 2 - Finley roots; 5, 6- Finley shoots; 3, 4 - 442 roots; 7, 8 - 443 roots. Odd numbered panels - untreated; even-numbered panels roots colonized with *Pseudomonas fluorescens*. Biotin internal standard - lower right corner of each panel (yellow-red).

Table 1. Functional identities of 41 genes represented by 95 oligonucleotides in a microarray interrogated with cDNA from hexaploid wheat

No. of Oligo-nucleotides[a]	GenBank No.	Gene
2	AB020958	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit
2	AF092524	Manganese superoxide dismutase
2	AF097363	Heat shock protein 101 (Hsp101b)
2	AF097659	Heat shock protein HSP26 (hsp26.6)
2	AF104108	Heat shock protein Hsp23.6
4[b]	AF126021	Homo sapiens B-cell receptor-associated protein BAP37 mRNA,
2	AF255053	Cold-responsive LEA/RAB-related COR protein (Wrab17)
2	AF387739	Leaf ascorbate peroxidase (WAPX1)
5	AJ249933	Puroindoline-a
2	BE403163	Actin
2	BE403755	CEO1A programmed cell death
3	BE405001	CEO1B programmed cell death
1	BE405525	BAX1 programmed cell death
4[b]	BE405591	DAD1A programmed cell death
2	BE406294	LPT1 Pr protein
4[b]	BE415173	DAD1B programmed cell death
2	BE423294	sucrose synthase II
4[b]	BE423527	alpha/beta gliadin
1	BE426368	alpha-tubulin3 (H. vulgare)
2	BE442962	LTP2 Pr protein
2	BE444854	sucrose-6F-phosphate phosphohydrolase
2	BE445579	COI1A JA Signaling
2	BE515427	LEA2
2	BE637556	Starch Branching Enzyme I
2	BF201050	sucrose phosphate synthase I (H. vulgare)
5[b]	BI479113	pMA1951 (ABA responsive)
2	D86327	Catalase
2	M16991	wheat alpha-amylase gene
2	M21812	Human (clone PWHLC2-24) myosin light chain 2
3	M94726	Protein kinase pKABA
4[b]	TAE010455	glutathione peroxidase
2	TAU55859	heat shock protein 80
2	TAU55860	heat shock protein 90
1	TAU69632	Cu/Zn superoxide dismutase (SOD1.2)
2	TC21853	cytosolic monodehydroascorbate reductase
2	TC26946	GSH-dependent dehydroascorbate reductase
2	TC26997	group 3 LEA
2	TC29145	Fe superoxide dismutase

2	TC31291	lysophospholipase
2	X58279	mRNA for heat shock protein 17.3

[a] The number of oligonucleotides included in a small microarray representing the indicated GenBank accession.
[b] Oligonucleotides representing the indicated GenBank accession included both sense and antisense oligonucleotides.

Table 2. Statistically significant[a] fold changes in expression levels of oligonucleotides representing the indicated genes following exposure to heat, cold, or root colonization by *Pseudomonas fluorescens* (bacteria).

GenBank or TIGR Accession Number and Name of Gene Represented	Wheat Accession[b]										
	442					443					Finley
	Roots			Shoots		Roots			Shoots		Roots
	Cold[c]	Heat[d]	Bacteria[e]	Heat[d]	Cold[c]	Cold[c]	Heat[d]	Bacteria[e]	Cold[c]	Heat[d]	Bacteria[e]
BE637556 Starch Branching enzyme I		3.1	1.9		1.4						
BE423294 sucrose synthase II									1.4		
BE444854 sucrose-6F-phosphate phosphohydrolase	1.2	1.5					1.3				
M16991Wheat alpha-amylase gene		1.5					2.4		1.1	1.8	1.2
AF092524 Manganese superoxide dismutase	0.7	0.6	0.8			0.7					
TAU69632 Cu/Zn superoxide dismutase	0.6				1.4	0.5					1.4
TC21853 cytosolic monodehydroascorbate reductase							1.2				
AF387739 Leaf ascorbate peroxidase									1.3		
TC26946 GSH-dependent dehydroascorbate reductase	0.7	1.3					0.7				
D86327.1 Catalase	0.6							1.2		1.4	
TAE010455 Glutathione peroxidase						0.6			1.2	1.4	1.3
X58279 Heat shock protein 17.3							1.6			1.5	
AF104108 Heat shock protein 23.6		1.3					1.2		1.4	1.5	
AF097659 Heat shock protein 26.6						0.6		1.2			0.8
TAU55859 Heat shock protein 80	1.2	1.5				0.7	1.2		1.2		
TAU55860 Heat shock protein 90	0.5			2.1	1.4	0.7		0.5	1.2		0.9
AF097363 Heat shock protein 101							1.2			1.1	
BE403755.1 CEO1A Programmed Cell Death		2.0					1.7				
BE405001.1 CEO1B Programmed Cell Death							0.6			0.8	
BE405011.1 CEO1C Programmed Cell Death								0.6	1.3	1.8	
BE405525.1 BAX1 Programmed Cell Death	0.5									1.3	
BE406294.1 LPT1 Pr Protein		0.4				0.9			1.5		
BE442962.1 LTP2 Pr Protein		2.4							1.1		
TC31291 lysophospholipase	3.0							1.6			
BE515427 LEA2	1.2	1.5					1.3				
TC26997 group 3 LEA	0.5							2.2			

AF255053 Cold-responsive LEA/RAB-related COR protein WRAB17	1.9					0.6			1.2		
BE415173.1 DAD1B Programmed Cell Death		1.3	0.8				1.2				0.8
BE405591.1 DAD1A Programmed Cell Death	0.4	0.3	0.7	1.8		0.9	0.7			1.7	0.7
M94726 pKABA					1.3	0.9			1.2	1.2	
AJ242716.1 Purindoline b	2.5	2.1	2.1		1.7		0.4	0.8	1.7	1.6	0.6
BE423527 Alpha/Beta gliadin					1.3				1.3	1.3	1.4
BE445579.1 COI1A JA Signaling	2.6	2.1	2.2		1.6		2.1				
BI479113 pMA1951	2.0	2.6			1.7			2.1	1.5	1.9	
AF126021 Homo sapiens B-cell receptor-associated protein BAP37	1.9	2.4					1.5		1.6		
M21812 Human myosin light chain 2	0.5	0.5				0.7	0.7				
Number of genes up-regulated	9	14	3	2	8	0	12	5	16	13	4
Number of genes down-regulated	9	4	3	0	0	11	5	3	0	1	5

[a]Statistical significance indicated by SAM software (Tuscher *et al.,* 2001).

[b]Wheat accession 442 and 443 are near-isogenic lines differing primarily at the vrn1a-Fr1 locus, *i.e.* spring wheat *vs.* winter wheat in otherwise identical genotypes.

[c] Cold - wheat seeds were germinated in darkness at 25^{o}C in plastic boxes on moist filter paper, allowed to grow for four days, were exposed to 2^{o}C for 1 h, and harvested immediately after cold treatment.

[d] Heat - wheat seeds were germinated as above, then were harvested immediately after a treatment of 50^{o}C for 1 h.

[e] Root-colonizing biocontrol bacteria - wheat seeds were coated with 10^5 CFU/ seed (Landa et al., 2002) of *Pseudomonas fluorescens* strain Q8r1-96 (Raaijmakers et al., 1999), germinated as above, and grown for four days prior to harvest.

Table 3. Numbers of genes showing coordinated regulation in winter wheat in response to three stress factors applied separately

	Number of genes responding to the stress factors			
Response[a]	Cold[b], Heat[c] and Bacteria[d]	Cold and Heat	Cold and Bacteria	Heat and Bacteria
Upregulated	7	9	2	1
Downregulated	2	3	2	1

[a]Statistically significant response indicated by SAM software (Tuscher *et al.,* 2001).

[b] Cold - wheat seeds were germinated in darkness at 25^{o}C in plastic boxes on moist filter paper, allowed to grow for four days, were exposed to 2^{o}C for 1 h, and harvested immediately after cold treatment.

[c] Heat - wheat seeds were germinated as above, then were harvested immediately after a treatment of 50^{o}C for 1 h.

[d] Root-colonizing biocontrol bacteria - wheat seeds were coated with 10^5 CFU/ seed (Landa et al., 2002) of *Pseudomonas fluorescens* strain Q8r1-96 (Raaijmakers et al., 1999), germinated as above, and grown for four days prior to harvest.

Table 4. Comparison of long oligonucleotide microarray results with quantitative real-time PCR results

Gene	Wheat accession[a]	Stress treatment[b]	Fold-change indicated by qPCR	Fold-change indicated by Microarray[c]
Manganese superoxide dismutase	442	Heat	0.7	0.6
		Cold	**1.6**	**0.7**
		Bacteria	0.9	0.8
	443	Heat	0.7	ns
		Cold	**1.0**	**0.7**
		Bacteria	1.2	ns
Glutathione Peroxidase	442	Bacteria	1.1	ns
	443	Bacteria	1.2	2.8
	Finley	Bacteria	1.4	3.0
Coi1	442	**Heat**	**0.7**	**2.1**
		Cold	1.2	2.6
		Bacteria	2.6	2.2
	443	**Heat**	**0.5**	**2.1**
		Cold	0.6	ns
		Bacteria	1.2	2.0
	Finley	Bacteria	1.1	2.2

[a]Wheat accession 442 and 443 are near-isogenic lines differing primarily at the vrn1a-Fr1 locus, *i.e.* spring wheat *vs.* winter wheat in otherwise identical genotypes; Finley is a winter wheat cultivar.
[b] Cold - wheat seeds were germinated in darkness at 25^{o}C in plastic boxes on moist filter paper, allowed to grow for four days, were exposed to 2^{o}C for 1 h, and harvested immediately after cold treatment; heat - wheat seeds were germinated as above, then were harvested immediately after a treatment of 50^{o}C for 1 h; bacteria - wheat seeds were coated with 10^5 CFU/ seed (Landa et al., 2002) of *Pseudomonas fluorescens* strain Q8r1-96 (Raaijmakers et al., 1999), germinated as above, and grown for four days prior to harvest.
[c]Changes were indicated as statistically significant by SAM software (Tuscher *et al.,* 2001).

Fig. 1

>Oligo23 gi|42601010|gb|AY533104.1| Triticum aestivum beta-expansin 2 (EXPB2) gene, complete cds Position=79-139 GC=0.65 Tm=80.12 Self-compl.=0.40
CGGCGCGTGCATCCCGAGGGTACCGCCGGGCCCCAACATCACCACCAACTACAACA
ACCA

Fig. 2